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Quantification of the Interactions between β-Lactoglobulin and Pectin through Capillary Electrophoresis Analysis

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Biopolymer interactions have many potential applications in pharmaceutical, cosmetic, nutraceutical, and functional food industries. Attractive interactions between proteins and polysaccharides can lead to the formation of complexes. Binding parameters of β-lactoglobulin (β-lg)/pectin complexes were determined using frontal analysis continuous capillary electrophoresis and the overlapping binding site model. At pH 4, approximately 23 β-lg molecules were cooperatively complexed on low-methoxyl pectin, where each β-lg molecule covered an average of 12 galacturonic acid residues. The calculated binding constant was 1431 M^{-1}. The interactions between pectin and four selected peptides located on the outer surface of the β-lg were investigated in order to identify which part of the protein was likely to interact with the pectin. The peptide β-lg 132−148, which corresponds to the α-helix zone, and the peptides β-lg 76−83, 41−60, and 1−14 would be involved in the interaction with the pectin.

KEYWORDS: β-Lactoglobulin; pectin; peptides; interactions; capillary electrophoresis

INTRODUCTION

The use of biopolymers such as polysaccharides and proteins is increasing in the pharmaceutical, cosmetic, nutraceutical, and functional food industries. Interactions between proteins and synthetic polymers have been largely investigated (*1*−*8*). Biopolymer interactions have been applied in the development of intestinal patches for drug-controlled delivery (*9*) and in polymeric nanoparticles used as carriers and protectors in oral vaccines and gene therapy (*10*).

β-Lactoglobulin (β-lg) is the major protein of bovine milk whey. This highly nutritive protein possesses all of the essential amino acids. The ability of β-lg to bind hydrophobic ligand, such as vitamins or fatty acids, has been extensively studied. Crystallographic studies have shown that molecules such as retinol, retinoic acid, palmitate, and cholesterol were able to bind in the central hydrophobic calyx of the β-lg (*11*−*14*). However, the biological function of β-lg is still unknown (*11*). The β-lg is a small globular protein of 18 350 Da. The β-lg has a well-known structure (*15*) containing mainly β-sheets, some β-turns, and one α-helix. The quaternary structure of β-lg is affected by pH conditions, mineral content, and temperature. The protein is monomeric at very acidic pH (i.e., below 3) and alkaline pH (i.e., above 8), dimeric at neutral pH, and found in both states between pH 3 and pH 6 (*16*). The presence of the octameric form around pH 4.5 is likely to be negligible (*16*, *17*). Pectin is a cell wall polysaccharide of plants generally extracted from apple pomace or citrus fruits. Pectin is a soluble fiber that reduces the risks of colon cancer (*18*). This anionic polysaccharide is essentially made up of D-galacturonic residues (D-galA). Some authors have investigated the interactions in β-lg and pectin mixtures in solutions (*19*) and in emulsions and gels (*20*, *21*).

Few studies have been carried out on the quantification of β-lg/polysaccharide interactions. Measuring the binding parameters that characterize protein/polysaccharide complexes is essential to accurately evaluate binding conditions. Biopolymer interactions can be difficult to quantify because of the heterogeneity of the natural material. Frontal analysis continuous capillary electrophoresis (FACCE) was developed to study interacting homogeneous molecular mass systems (*8*).

In this study, FACCE was used to separate the noncomplexed (free) β-lg from the pectin and the β-lg/pectin complexes, to plot the binding isotherms, and to calculate the binding stoichiometry of the β-lg/pectin complexes. Results obtained with FACCE were treated with the overlapping binding site model (*22*). This model was used to calculate the binding constant, the cooperativity parameter, and the binding site size on the pectin.

Previous work has also revealed that interactions between β-lg and pectin occurred via charged molecules and hydrogen bonding (*23*). To identify potential interacting zones on the β-lg, the interactions between four peptides on the outer surface of β-lg and pectin were probed (**Figure 1**). The peptides β-lg 1−14 and 76−83 were selected because of their positive net charge at acidic pH. β-lg 41−60 is a tryptic peptide with good emulsifying properties (*25*, *26*). The peptide β-lg 132−148, which is part of the α-helix, was shown to interact with the acacia gum (*27*).

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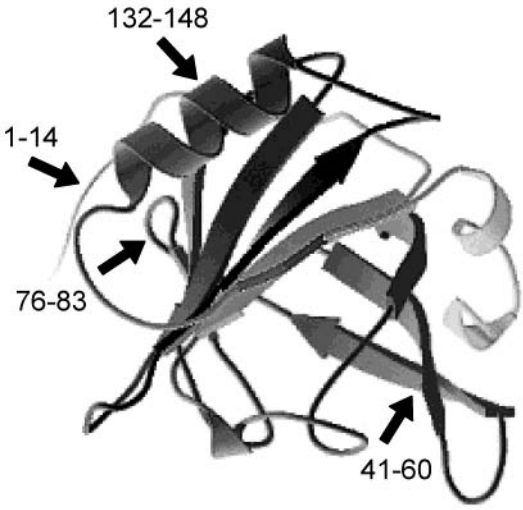

Figure 1. Localization of the selected peptides on the β-lg molecule. The β-lg structure was from Qin et al. (*21*) and was reproduced (3BLG image) from the Protein Data Bank Server.

MATERIALS AND METHODS

Materials. Bovine β-lg AB 95% pure protein (3× crystallized) was purchased from Sigma-Aldrich Canada Ltd. (Oakville, ON, Canada) and used without further purification. Peptides β-lg 1−14 (LIVTQTMKGLDIQK), 41−60 (VYVEELKPTPEGDLEILLQK), 76−83 (TKIPAVFK), and 132−148 (ALEKFDKALKALPMHIR) were synthesized by the Eastern Quebec Proteomics Core facility (Fmoc technology) and used as received. Low-methoxyl (LM) pectin (DE 28.3%, apparent molecular mass 94.3 kDa) and high-methoxyl (HM) pectin (DE 73.4%, apparent molecular mass 105 kDa) were provided by Hercules Copenhagen A/S (Copenhagen, Denmark). All reagents were of analytical grade and used as received.

Preparation of Solutions. β-lg, peptides β-lg 1−14, 41−60, 76−83, and 132−148, as well as LM and HM pectin were dispersed in 5 mM sodium phosphate buffer and stored overnight at 4 °C to ensure the complete hydration of molecules. The pectin and protein or peptide solutions were mixed at molar ratios between 4:1 and 40:1 for protein/pectin solutions and between 5:1 and 95:1 for peptide/pectin solutions keeping the pectin concentration constant at 5 μM. All calculations involving β-lg were made using the monomeric form as the unit. The pH of mixed protein/pectin solutions was adjusted to 4, 5, or 6 and pH 4 for the peptide/pectin solutions. Measures on protein/pectin solutions at pH 5 in 50 mM sodium phosphate buffer were also carried out. The mixtures were stored overnight at 22 °C for interactions between molecules to occur.

FACCE. Protein/polyelectrolyte interactions were studied with FACCE (*1*, *8*). FACCE is used to study slow binding kinetic systems since the equilibrium in the sample vial is reached prior to the analysis. FACCE is an interesting technique for binding studies, because it is not affected by the Donnan exclusion effect observed with ultrafiltration membranes (*23*, *28*, *29*). FACCE differs from conventional CE methods as it provides continuous sampling throughout the analysis. Thus, each charged molecule is detected as a continuous plateau instead of a peak. Generally, one or two plateaus are obtained since the polysaccharide is often undetected. Under a positive polarity (i.e., anode to cathode migration), the first eluting plateau corresponds to the free protein, which is positively charged. The second plateau is added to the first and corresponds to the protein complexed with the polysaccharide (*8*). The concentration of the free protein was determined using a calibration curve from known β-lg concentrations.

FACCE was carried out on a Hewlett-Packard HP^{3D}CE provided with a UV diode array detector (Agilent Technologies Inc., Mississauga, ON, Canada). The bare-fused silica capillary used (i.d. 50 mm) had a total length of 33 cm and an effective length of 24.5 cm (Agilent Technologies Inc.). The temperature of the cassette was maintained at 22 ± 0.1 °C. The detection wavelength was 200 nm to avoid interference by pectin absorbance. Before each analysis, the capillary was flushed with 0.1 N NaOH for 3 min and equilibrated for 5 min with 5 or 50 mM sodium phosphate buffer at sample pH. The sample was introduced electrokinetically in the capillary (intrinsic to the FACCE method) for 30 min with 9 kV applied with positive polarity. The capillary was rinsed daily with 0.1 N NaOH for 15 min, followed by water for 5 min, and then dried.

Ultrafiltration. Interactions between peptides and LM pectin were studied with an ultrafiltration method previously described (*23*, *28*). Peptide/pectin mixtures were shaken gently; 100 μL of the mixtures was ultrafiltrated by centrifugation at 10 000*g* for 25 min at 22 °C through 10 kDa MWCO Microcon devices (Millipore, Bedford, MA). The membrane retained the pectin and the complexes, while the free peptides passed through the membrane. The free peptides recovered in the ultrafiltration permeate were evaluated with the bicinchoninic acid assay using known concentrations of corresponding peptides in order to obtain the standard curves (*30*).

Calculation of the Binding Parameters. Given the nonspecific nature of the complexation and the large size of the ligand (protein) relative to the macromolecule (pectin), the mathematical model used had to take into account the possibility of overlapping binding sites. Namely, the fact that β-lg could occupy more than one binding site on the pectin had to be considered so as to prevent any overestimation of the potential binding sites on the pectin. According to the binding model of McGhee and von Hippel (*22*), complexation between ligand and macromolecule, including ligand−ligand interactions, can be expressed as

$$\frac{\nu}{L} = K(1 - n\nu) \cdot \left(\frac{(2\omega - 1)(1 - n\nu) + \nu - R}{2(\omega - 1)(1 - n\nu)}\right)^{n-1} \cdot \left(\frac{1 - (n + 1)\nu + R}{2(1-\text{-}\, n\nu)}\right)^{2}, R = \sqrt{(1 - (n + 1)\nu)^2 + 4\omega\nu(1 - n\nu)}$$

where L is the free ligand concentration (M), K is the binding constant (M^{-1}), n is the average size of the binding site (D-galA residues covered with a β-lg monomer), ν is the binding density (number of β-lg complexed per D-galA residue), and ω is the cooperativity parameter (unitless). The cooperativity parameter assesses ligand−ligand interactions. It has a negative value when the interactions are repulsive or anticooperative, a positive value for attractive or cooperative interactions, and is zero when no interactions are involved between ligand molecules. The mathematical model could not be used to determine if more than one amino acid zone on the β-lg was involved in the pectin interaction. The limitation of the overlapping binding site model was circumvented by studying the interactions between the four selected peptides β-lg and LM pectin.

Results obtained with FACCE and UF were used to obtain the binding isotherms of the β-lg or peptide/LM and HM pectin mixtures through plotting the binding density (ν) against the free β-lg concentration (L). The L parameter could be directly calculated from the FACCE and UF results. Given that the LM and HM pectin used contained 80% of D-galA and that their apparent molecular masses are known, their average D-galA content was approximated to 420 and 507 residues per LM and HM pectin molecule, respectively. Therefore, the ν parameter could be obtained from the total β-lg or peptide molecules complexed divided by 420 and 507. The saturation of pectin estimated from the binding isotherms made it possible to calculate the binding stoichiometry (N) expressed as β-lg or peptide molecules per pectin molecule.

The binding constant, cooperativity parameter, and binding site could be determined by the nonlinear least-squares fitting of ν/L plotted against ν. The reproducibility of the binding isotherms was verified through experimental points carried out in duplicates. Binding isotherms presented are typical replicates of each experiment. The curve fitting and the calculation of standard errors on the binding parameters (N, K, ω, and n) were carried out with the JMP IN software (SAS Institute Inc, U.S.A.).

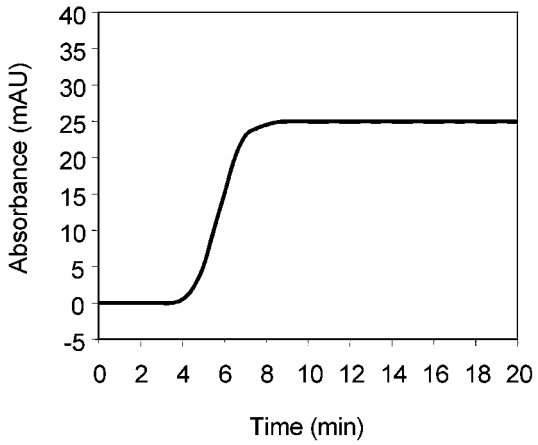

Figure 2. Typical electropherogram obtained for the β-lg/LM or HM pectin complexes at 200 nm.

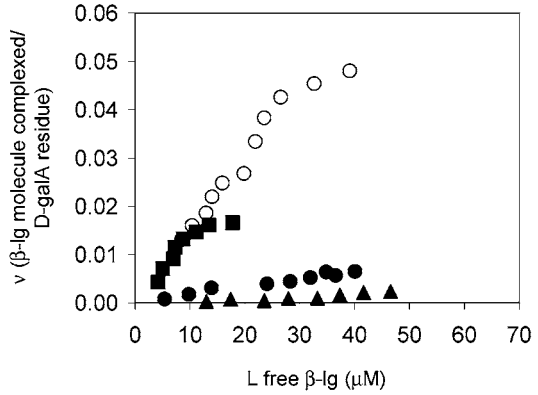

Figure 3. Binding isotherms of β-lg/LM pectin mixtures in 5 mM phosphate buffer at pH 4 (○), 5 (■), and 6 (▲) and in 50 mM phosphate buffer at pH 5 (●).

RESULTS

Figure 2 shows a typical electropherogram obtained for the β-lg-containing solutions at all experimental conditions. The single plateau corresponds to the UV absorbance of the free β-lg, which has a net positive charge below its isoelectric point (IEP) (5.1) and positively charged "patches" between pH 5.1 and pH 6 (*23*). Similar results obtained with UF (results not shown) confirmed that the single plateau was not caused by similar migration times of the free and complexed protein. The absence of the second plateau is explained by the lack of positive charges on β-lg/pectin complexes, which prevented their introduction into the capillary.

The binding isotherms of the β-lg/LM pectin mixtures at pH 4, 5, and 6 and in 50 mM sodium phosphate buffer at pH 5 are illustrated in **Figure 3**. The amount of β-lg complexed with LM pectin was negligible at pH 6. At pH 5, the amount of β-lg/LM pectin complexes dramatically decreased as the phosphate buffer concentration was increased from 5 to 50 mM. At this pH, there was about 0.018 and 0.003 β-lg molecule complexed per D-galA residue for a free β-lg concentration around 15 μM in 5 and 50 mM phosphate buffer, respectively. At pH 4 in 5 mM phosphate buffer, 0.025 monomer of β-lg was complexed on each D-galA residue, corresponding to the appearance of a small plateau at free β-lg values between 15 and 20 μM. From these binding isotherms, the saturation of a D-galA residue with β-lg could be approximated with the end of the curves. At this point, the higher amount of complexed β-lg with an increasing protein/pectin ratio was almost stopped indicating pectin saturation. A D-galA residue of the LM pectin reached saturation at 0.019 and 0.055 β-lg molecule in 5 mM phosphate buffer at pH 5 and 4, respectively. At pH 5 in a 50 mM phosphate buffer, the saturation of a D-galA residue was reached with 0.008 β-lg molecule.

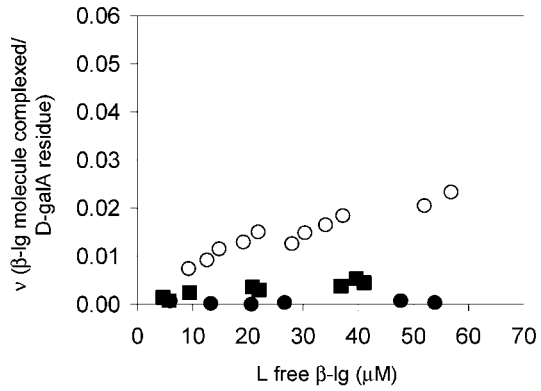

Figure 4. Binding isotherms of β-lg/HM pectin mixtures in 5 mM phosphate buffer at pH 4 (○) and 5 (■) and in 50 mM phosphate buffer at pH 5 (●).

Table 1. Binding Parameters of β-lg/LM and HM Pectin Complexes Calculated with the Overlapping Binding Site Model[a]

mixtures	pH	buffer (mM)	*N*	*K* (M^{-1})	ω	*n* (D-galA residues)
β-lg/LM pectin	4	5	23	1431	18	12
	5	5	8	547	242	50
	5	50	3	166	12	11
	6	5	2	40	20	19
β-lg/HM pectin	4	5	13	857	15	20
	5	5	2	235	18	68

[a] Standard error is lower than 15%. *N*, binding stoichiometry; *K*, binding constant; ω, cooperativity parameter; *n*, binding site size.

Figure 4 shows the binding isotherms obtained with the HM pectin. At free β-lg concentration around 15 μM in 5 mM phosphate buffer, an average of 0.002 and 0.015 β-lg molecule is complexed on each D-galA residue at pH 5 and 4, respectively. There were no more β-lg molecules complexed at pH 5 with a 50 mM phosphate buffer concentration. At pH 5 and 4, the D-galA residue reached saturation at 0.003 and 0.025 β-lg molecule, respectively. No significant complexation was noticed at pH 6 (not shown).

Given that there were approximately 420 and 507 D-galA residues per LM and HM pectin molecules, respectively, the binding stoichiometry (*N*) of complexes could be calculated using the pectin saturation values presented above and reported in **Table 1**. At pH 5 and 4, approximately 23 and eight β-lg monomers were complexed per LM pectin molecule and 13 and two β-lg monomers were complexed on the HM pectin. The binding constants (*K*) show that LM pectin is more reactive than the HM pectin as was the case with the binding isotherms. The binding constant decreased at higher pH values. The positive cooperativity values (ω) indicate an attractive interaction between β-lg molecules, especially as they complex with the LM pectin at pH 5. Consequently, the β-lg molecules would be complexed on the pectin, preferably near one another. The binding site size values *n* on LM pectin show that a β-lg monomer covers about 15 D-galA residues, except at pH 5, where the β-lg covered approximately 50 residues. Under the same conditions, the HM pectin would have a binding site

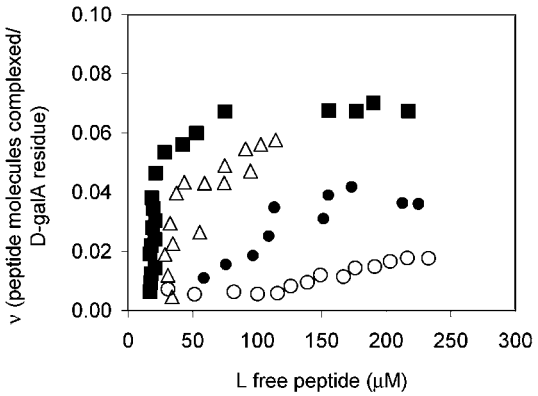

Figure 5. Binding isotherms of β-lg peptides 1–14 (○), 41–60 (●), 76–83 (■), and 132–148 (△)/LM pectin complexes at pH 4 in 5 mM sodium phosphate buffer.

Table 2. Binding Parameters of the β-lg Peptides 1–14, 41–60, 76–83, or 132–148/LM Pectin Complexes at pH 4 in 5 mM Phosphate Buffer Calculated with the Overlapping Binding Site Model[a]

peptide/ LM pectin	N	K (M^{-1})	ω	n (D-galA residues)
1–14	8	90	7	12
41–60	17	144	46	8
76–83	31	245	207	15
132–148	25	163	118	16

[a] Standard error is lower than 15%. N, binding stoichiometry; K, binding constant; ω, cooperativity parameter; n, binding site size.

slightly larger than that of the LM pectin. An increasing concentration of the phosphate buffer leads to lower values of binding stoichiometry, binding constant, cooperativity, and binding site size.

To identify the potential reactive zones on the β-lg, assays were carried out under the most reactive conditions, i.e., with LM pectin at pH 4. The bare-fused silica capillary could not be used with peptides because of serious migration difficulties and lack of reproducibility. Furthermore, the peptides were analyzed with a poly(vinyl alcohol)-coated capillary (Agilent Technologies Inc.) to prevent interactions between the negative silanol groups on the capillary wall and the positive charges of the peptides. However, no satisfactory signal could be obtained, probably from the lack of eletroosmotic flow. Therefore, the ultrafiltration technique was used to study the interactions between the four selected peptides (i.e., β-lg 1−14, 41−60, 76−83, and 132−148) and the LM pectin. From the free peptide concentrations L, the binding density values ν could be determined. Binding isotherms obtained by means of plotting the binding density against the free peptide concentrations are presented in **Figure 5**.

At a free peptide concentration around 50 μM, an average of 0.005, 0.01, 0.04, and 0.06 molecules of the peptides β-lg 1−14, 41−60, 132−148, and 76−83, respectively, were complexed on each D-galA residue. From these binding isotherms, the approximated saturation of a D-galA residue with peptides β-lg 1−14, 41−60, 132−148, and 76−83 were, respectively, 0.02, 0.04, 0.06, and 0.07 molecule. The stoichiometry values of the peptide/LM pectin complexes are presented in **Table 2**. The peptides β-lg had their binding constant values (K) in ascending order: 1−14, 41−60, 132−148, and 76−83. The cooperative values (ω) showed attractive interactions between the peptides complexed on the LM pectin. The β-lg peptides 1−14, 76−83, and 132−148 covered about 15 D-galA residues, whereas the β-lg peptide 41−60 covered eight D-galA residues. Although the binding constants for the peptides β-lg 41−60 and 132−148 were almost equivalent, the stoichiometry and the cooperative behavior for β-lg 41−60 are not as pronounced as those obtained for the peptide β-lg 132−148.

DISCUSSION

The β-lg/pectin complexes are maintained by ionic interactions and hydrogen bonding (*23*). Thus, the pectin is not complexed through the hydrophobic zone of the protein as was observed for small ligands, such as retinol, retinoic acid, palmitate, and cholesterol (*11*). The stoichiometry of the complexes decreases with increasing pH values. The negative net charge appearing above the IEP of the protein (5.1) limits the interaction with the negatively charged pectin. Hence, the size of complexes is greatly increased below the IEP since the protein bears a positive net charge. The pH and ionic strength are parameters that mainly influence electrostatic interactions (*31*−*33*). The small plateau noticed at pH 4 for the β-lg/LM and HM pectin mixtures may indicate the need of a critical binding stoichiometry in order to get a higher degree of complexation. According to the Tainaka theory (*35*, *36*), protein and polyelectrolyte complexation is a two step process. The first step corresponds to the formation of intrapolymer complexes. An intrapolymer complex is a molecular assembly made up of a few β-lg molecules bonded on a pectin molecule. The size of an intrapolymer complex is in the range of the pectin molecule, which is the largest of the assembly. The second step is the aggregation of the intrapolymer complexes, which produces interpolymer complexes. At pH 5, eight β-lg monomers were complexed with LM pectin. This value corresponds to the binding stoichiometry of β-lg/LM pectin intrapolymer complexes at pH 4 measured with isothermal titration calorimetry (ITC) (*34*). The amount of β-lg (18 kDa) complexed on each LM pectin molecule (94.3 kDa) at pH 4 is quite high considering the relative molecular masses of the molecules. Under the same conditions, ITC revealed that interpolymer complexes had a binding stoichiometry of 25 β-lg monomer/LM pectin (*34*). Consequently, β-lg/LM pectin intrapolymer complexes probably aggregate in interpolymer complexes between pH 4 and pH 5.

The electrostatic nature of the interactions is confirmed by the screening effect on both β-lg and pectin charges as the buffer concentration increases from 5 to 50 mM. The increased ionic strength decreases the initial pH of the intrapolymer and interpolymer complex formation (*23*, *37*, *38*). Therefore, an increase in ionic strength reduces the amount of interpolymer complexes and consequently the binding stoichiometry and binding constants. Even though HM pectin had a higher molecular mass than the LM pectin, there were always fewer amounts of complexed protein molecules as well as lower binding constants. This is explained with the higher amounts of carboxyl groups on the LM pectin involved in the interaction with the β-lg. FACCE has shown that 13 and 23 β-lg monomers were complexed at pH 4 on the HM pectin and LM pectin, respectively. The stoichiometry value obtained with HM pectin is almost half the value obtained previously with ITC (*34*). This difference could be explained by the polarizing effect of the electric field used in CE that could decrease, to some extent, hydrogen bonding in the complexes. Hydrogen bonding was shown to be more important in the complexes containing HM pectin as compared to those containing LM pectin (*23*). Interactions were stronger with lower pectin DE, because of

the higher amount of carboxyl groups responsible for the interactions with the β-lg. It was previously shown with ultrafiltration that LM pectin was more reactive with β-lg than with HM pectin (*23*).

The complexation may reveal a cooperative behavior between protein molecules (*39*), which can be detected from both the curvature of the binding isotherm and the fitting values. It was obvious from the fitting values that the β-lg molecules complexed on the LM pectin were cooperative. The pH and ionic strength had a moderate to high effect on the cooperative behavior of β-lg/LM or HM pectin mixture complexes. The higher cooperativity in the β-lg/LM pectin mixture at pH 5 in the 5 mM phosphate buffer can be explained by the protein's weak net charge around its IEP, which promotes β-lg/β-lg interaction. The lower cooperative behavior observed at pH > IEP is explained by the net negative charge of the protein resulting in Coulombic repulsion between the β-lg molecules. Moreover, the lower cooperativity observed at pH 4 is also explained by repulsive forces between the positively charged β-lg molecules. The screening effect by the higher buffer concentrations lowered the attraction between the molecules and then the cooperativity. The noncooperative behavior of β-lg with HM pectin at pH 5 can be explained by the lower amounts of carboxylic groups. These groups are sufficiently dispersed on the backbone of the pectin to prevent the protein molecules from complexing near one another. A previous study showed that the β-lg was highly cooperative ($\omega = 128$) with sodium polystyrene sulfonate (NaPSS) at pH 7.5 in 100 mM phosphate buffer and least cooperative ($\omega = 3$) at pH 6.7 in 50 mM of the same buffer (*40*).

There is a tendency for the HM pectin to have a larger binding site size. This trend is hard to explain since β-lg was expected to occupy the same volume while complexing on LM or on HM pectin. The larger binding site size observed at pH 5 for both LM and HM pectin can be explained by β-lg/β-lg interactions around protein's IEP. A β-lg molecule interacting with other protein molecules may occupy a larger space on the pectin. This is in agreement with the higher cooperativity observed at this pH value for LM pectin. The binding site size of the β-lg/sodium poly(2-acrylamido-2-methylpropanesulfonate) complexes was dependent on both pH and ionic strength (*1*). However, the binding site size of the β-lg/NaPSS complexes was independent of the pH and ionic strength because of the hydrophobic interactions involved (*40*).

The negatively charged macromolecules would interact with β-lg at more than one binding site (*41*). Consequently, the pectin is likely to interact through more than one amino acid zone on the β-lg. Circular dichroism revealed the involvement of the α-helix of β-lg in the interaction with acacia gum (*28*). However, this technique could not be used to identify interacting zones having no particular secondary structures. The potential interacting zones on the β-lg are supposed to be positively charged and located on the outer surface of the protein.

Ultrafiltration and the overlapping binding site model were used to determine the binding parameters of the four selected β-lg peptides/LM pectin complexes. The β-lg peptides had their stoichiometry, binding constants, and cooperativity parameter in decreasing order: 76−83, 132−148, 41−60, and 1−14. The primary structures of the peptides can explain these reactivity differences. The most reactive β-lg peptide is the 76−83. Its IEP (i.e., 10.0) was calculated with the ExPASy server. At pH 4, this peptide has a 2+ net charge (from the Lys_{77} and Lys_{83} residues). The second most reactive peptide was the β-lg 132−148 with a calculated IEP of 9.70. This peptide has a 3+ net charge at pH 4 with five positive charges coming from the Lys_{135}, Lys_{138}, Lys_{141}, His_{146}, and Arg_{148} residues and two negative charges originating from the Glu_{134} and Asp_{137} amino acids that are still not protonated at pH 4 (*41*). According to its net charge at pH 4, the peptide β-lg 132−148 should be the most reactive one. However, this peptide has a higher molecular mass (1981 Da) than the sequence β-lg 76−83 (903 Da) and the Phe_{136} and Ala_{139} reactive residues are in a hydrophobic patch (*42*). The peptide β-lg 41−60 was the third most reactive peptide followed by β-lg 1−14, being the least reactive one. The peptide β-lg 41−60 had a calculated IEP of 4.25 and a −1 net charge at pH 4 resulting from two positively charged Lys_{47} and Lys_{60} amino acid residues and from three negatively charged Glu_{51}, Asp_{53}, and Glu_{55} amino acid residues (*41*). The peptide β-lg 1−14 had a calculated IEP of 8.59 and a +1 net charge due to the protonated Lys_8 and Lys_{14} residues and the Asp_{11} residue that is still ionized at pH 4 (*41*). Given their net charge at pH 4 and hydrophobicity, the peptide β-lg 1−14 was expected to be more reactive than the 41−60. However, this latter peptide is believed to interact with pectin through hydrogen bonding with its four glutamic acid residues, especially Glu_{51} and Glu_{55}, which are still ionized at pH 4.

The binding site sizes on the LM pectin were around 15 D-galA residues for peptides β-lg 1−14, 73−83, and 132−148 and eight D-galA residues for peptide β-lg 41−60. The lower binding site size of the β-lg 41−60 can be explained by the Pro_{48} and Pro_{50} residues that possibly induced some turns in the peptide. Therefore, both lysine residues could have gotten closer and lowered the binding site size or the turn could have limited the interaction with the pectin to one lysine residue.

Results show that β-lg would interact on two zones containing first the peptides β-lg 1−14, 41−60, and 73−76, because of their proximity on the tertiary structure of the β-lg, and then through the β-lg 132−148, which corresponds to the α-helix zone. However, the binding of pectin with native β-lg may be different from what was observed with the synthesized peptides β-lg 41−60 and 132−148, because of the possible lack of secondary structures and the greater accessibility of such short amino acid sequences. The dimer contact area involving the Asp_{33}, Ala_{34}, and Arg_{40} residues on both monomers could influence the binding site (*43*). The amounts of β-lg dimers increase with higher pH values (i.e., from 4 to 7) and with ionic strength (*16*, *43*). Consequently, β-lg and pectin interactions may have been overestimated, especially at higher pH values. However, the weak interaction measured between β-lg and pectin at pH 6 and the use of a low buffer concentration (5 mM) would have reduced the influence of β-lg dimeric form in the results presented. Binding site sizes and stoichiometry values calculated on the LM pectin for the peptides were generally comparable to those obtained for the β-lg. This suggests that the β-lg could probably complex on the pectin through the four selected peptides studied.

CONCLUSION

FACCE used with the overlapping binding site model is an efficient tool to carry out binding studies involving biopolymers such as proteins and polysaccharides. However, studies with peptides should be performed with coated capillaries to prevent their interaction with the capillary wall. FACCE is an inexpensive technique, because it requires only a small sample and no UF devices. FACCE was successfully used to obtain quantitative data on the β-lg/pectin complexes. However, the effect of the electric field on the dissociation of weak electrostatic complexes

should be further investigated. The higher amounts of carboxyl groups enabled the LM pectin to complex more proteins than HM pectin. The β-lg could possibly complex on pectin through its amino acid zones 76−83, 132−148, 41−60, and 1−14.

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